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Xsox17α and -β Mediate Endoderm Formation in Xenopus

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Summary

We have isolated two Xenopus relatives of murine *Sox17* expressed in gastrula presumptive endoderm. *Xsox17*α and -β expression can be induced in animal caps by activin, but not by FGF. Ectopic expression of these genes in animal caps induces the expression of endoderm markers; this induction is blocked by overexpression of a fusion of the Xsox17β HMG domain to the Drosophila Engrailed repressor domain, as is induction of endoderm markers by activin and the expression of endodermal markers in whole embryos and isolated vegetal poles. These experiments, as well as the effects of the mRNAs on embryo phenotypes, suggest that the *Xsox17* genes mediate an activin-induced endoderm differentiation pathway in animal caps and are involved in normal endoderm differentiation in embryos.

Introduction

In Xenopus, the endoderm forms from the yolky vegetal pole of the embryo, whereas the mesoderm develops from a ring around the equator, which is enveloped on both the inside and the outside by the presumptive endoderm, as shown in Figure 2B (Keller, 1991). The animal cap tissue contains the future ectoderm, which develops into epidermis and nervous tissue. During gastrulation, the vegetal cells internalize and elongate with the body axis, developing into gut, liver, pancreas, and lungs.

Although there is a considerable body of work on how the mesoderm and nervous system develop, the endoderm has received relatively little attention, partly through the lack of molecular markers and because the morphological differentiation of endoderm in the absence of mesoderm is poor. Our understanding of mesoderm induction has been facilitated by the fact that the animal cap may be induced to form mesoderm by TGFβ and FGF family members and then patterned by other molecules, such as the Wnts and BMP (Kessler and Melton, 1994). The use of various interfering constructs has shown that members of these families of inducers are naturally involved in forming the equatorial annulus of mesoderm. For example, the effects of dominant negative activin receptors, which promiscuously block signaling through TGFβ family receptors, indicate that the initiation of mesoderm formation depends on a TGFβ family ligand (Vg1 or some other family member, but not activin) emanating from the vegetal pole (Hemmati-Brivanlou and Melton, 1992; Kessler and Melton, 1994; Schulte-Merker et al., 1994). At a later stage, signals originating in the dorsal organizer region (e.g., noggin, chordin, and nodal-related molecules) and local signaling molecules in the ventral and lateral equatorial region (BMP4, Xwnt8) pattern the dorsal–ventral axis (Lemaire, 1996; Lemaire and Kodjabachian, 1996). Some of these molecules have multiple roles; for example, BMP4 represses the formation of nervous tissue by the animal cap and permits epidermal differentiation (Hemmati-Brivanlou and Melton, 1997).

Vegetal pole cells are committed to form endoderm from the early gastrula stage onward, as are blastula vegetal cells cultured to gastrula stages, but before gastrulation their fate can be altered by changing their position in the embryo (Wylie et al., 1987). Wylie et al. therefore postulated that the formation of endoderm is a cell-autonomous process, but one that can be altered up to gastrulation. More recently, these observations have been supported by experiments in which isolated vegetal poles from blastula embryos were cultured and endodermal marker expression was assayed. The mRNAs encoding the midgut marker Xlhbox8, the intestinal marker intestinal fatty acid binding protein or IFABP (Gamer and Wright, 1995; Henry et al., 1996), and the gut lumen marker detected by the monoclonal antibody 4G6 (Jones et al., 1993) are all expressed in isolated vegetal poles. The information to support such autonomous differentiation is likely to be of maternal origin and could include either cell autonomous molecules or those establishing a local signaling environment favoring endodermal differentiation.

Nothing is known of the way that cell autonomous molecules (e.g., transcription factors) promote endodermal development at blastula and gastrula stages, but it has been shown that in animal cap assays the mesoderm inducer activin is capable of inducing the endodermal markers mentioned above (Jones et al., 1993; Gamer and Wright, 1995; Henry et al., 1996), as well as *Mix1* (Rosa, 1989) and *HNF1*α (Weber et al., 1996). It has also been shown that inhibition of BMP signaling at the receptor, or at the ligand level by the mesoderm dorsalizers noggin and chordin, induces animal caps to express *Endodermin* and *Xlhbox8* (Sasai et al., 1996). Any of the signals mentioned above could be involved in the intrinsic ability of the vegetal pole to form endoderm, since a signal of the activin class (possibly Vg1) is generated by the vegetal pole and the organizer molecules are present in dorsal vegetal cells.

In order to shed further light on the earliest events of endoderm development, we have endeavored to find molecules that are important in this process by using a subtractive hybridization approach to isolate mRNAs restricted to the vegetal pole at the early gastrula stage (Lukyanov et al., 1994). We have cloned two SOX genes (*Xsox17*α and -β) most closely related to murine *Sox17*. They are expressed throughout the entire endoderm region at the gastrula stage and can be induced by activin, but not FGF, in animal cap assays. Overexpression of *Xsox17*α or -β in animal caps can specifically

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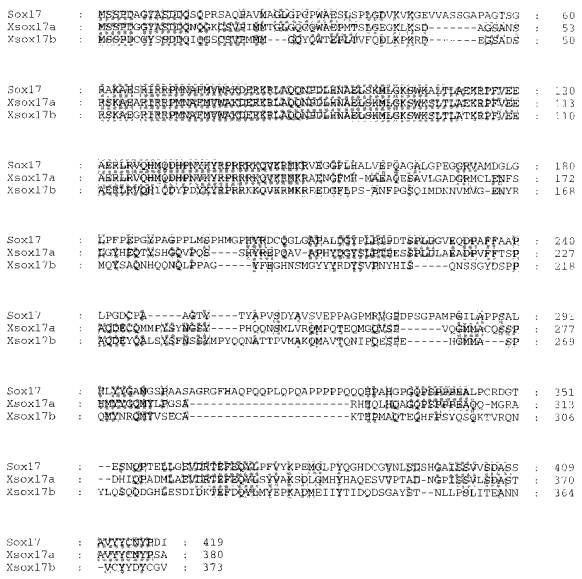

Figure 1. Comparisons of Murine Sox17, Xsox17α, and -β

The sequence comparison was made using the PILEUP program of the GCG package (Genetics Computer Group Version 8, 1994). Identical bases are shaded. Xsox17α is 50% identical and 63% similar to Sox17. It is 47% identical and 64% similar to Xsox17β. Xsox17β is 33% identical and 48% similar to Sox17. The HMG domains are 90% identical among all three.

induce the expression of the endodermal markers *Endodermin* (Sasai et al., 1996), *HNF1*β (Demartis et al., 1994), *IFABP* (Shi and Par Hayes, 1994; Henry et al., 1996), and *Xlhbox8* (Gamer and Wright, 1995). Furthermore, we show that by expressing a fusion of the Engrailed repressor to the HMG domain of the *Xsox17*β gene we can specifically prevent the induction of endodermal marker genes by activin and reduce endodermal markers in vegetal poles and whole embryos. Thus, *Xsox17*α and -β are both sufficient and necessary for endoderm formation in the Xenopus embryo.

Results

Cloning of Two Endoderm-Specific SOX Genes Closely Related to Murine *Sox17*

We constructed a probe from stage 10.5 gastrula–poly(A)$^+$ RNA in which animal hemisphere sequences were subtracted from vegetal. The subtraction methodology involves suppression of PCR amplification of those sequences that are in common (Lukyanov et al., 1994). We screened a vegetal pole library in λZAPII with the subtracted probe and isolated two different cDNAs containing the HMG domain characteristic of SOX genes. Probing Northern blots with both revealed single bands of approximately 1.6 kb (Figure 3A). Searching of the databases with the BLAST program (Altschul et al., 1990) indicated that they were both most closely related to murine *Sox17* (Figure 1).

The SOX subclass of proteins containing the HMG DNA binding domain are defined as showing at least 50% identity or 60% conservation within this region (Prior and Walter, 1996). The two cDNA clones we have identified fulfill these criteria and are most closely similar to murine *Sox17*, both within and outside the HMG domain (Figure 1). We have therefore called these genes *Xsox17*α and -β. There has been a tetraploidization event in the recent history of Xenopus laevis so that most alleles are present as duplicates, called pseudoalleles, but *Xsox17*α and -β are too divergent to be pseudoalleles (Bisbee et al., 1977; Knochel et al., 1986).

On the basis of the similarity of their HMG domains, SOX genes have been classified into six groups (Prior and Walter, 1996). *Xsox17*α and -β fall into a subgroup of SOX genes including *Sox7*, *Sox17*, and *Sox18*, of which *Sox17* and *18* have been shown to contain activation domains (Hosking et al., 1995; Kanai et al., 1996). The HMG domains of *Sox17*, *Xsox17*α, and -β are very similar, sharing over 90% identity, but *Xsox17*β is the most divergent, particularly in helix three. It is possible that the differences are important in DNA binding specificity, although the effects of the genes are indistinguishable in the experiments reported here.

Expression of *Xsox17*α and -β during Development

Neither *Xsox17* gene shows any maternal expression, and both transcripts become detectable in the late blastula. For both, a single 1.6 kb transcript is detectable by Northern blotting (Figure 3A). *Xsox17*α was expressed at all stages from 10 to 35, but *Xsox17*β becomes undetectable during tailbud stages. Quantitative RT-PCR showed that in early gastrulae both are expressed in the vegetal pole and equatorial region, but not at all in the animal hemisphere. This is exactly as expected from the screen in which they were cloned, that is, using a vegetal probe from which animal cap sequences were subtracted. More surprisingly, some transcripts were detectable in the animal pole of the late blastula, and this low level persisted in animal caps isolated from blastulae and cultured on to gastrula (Figure 3B).

*Xsox17*α and -β Are Expressed in the Entire Endoderm of the Gastrula Embryo

In situ hybridization was carried out to a variety of embryonic stages to determine the expression pattern of the two genes. These patterns are very similar except that, as expected from the Northern analysis, *Xsox17*α is expressed for longer than *Xsox17*β. Transcripts are primarily nuclear at stage 10, and by stage 10.5 expression is visible in a superficial ring around the blastopore and throughout the vegetal region (Figures 2A–2D), both regions fated to be endoderm (Figure 2B; Keller, 1991). There is no staining in the presumptive mesoderm (Figure 2D), the region that expresses *Xbra* (Figure 2E). The expression remains in the endoderm throughout gastrulation and neurulation, but at tailbud stages the two expression patterns begin to differ: *Xsox17*α is strongly expressed in the entire endoderm at stage 26 (Figure 2F), whereas *Xsox17*β is present at scarcely detectable levels, and by stage 35 it is absent. In the tailbud embryo (Figure 2G), sectioning shows that the dorsal line of expression is in the dorsal wall of the gut and not at all in the notochord. By stage 35, *Xsox17*α transcripts become restricted to the most posterior endoderm and a ring around the opening of the liver diverticulum into

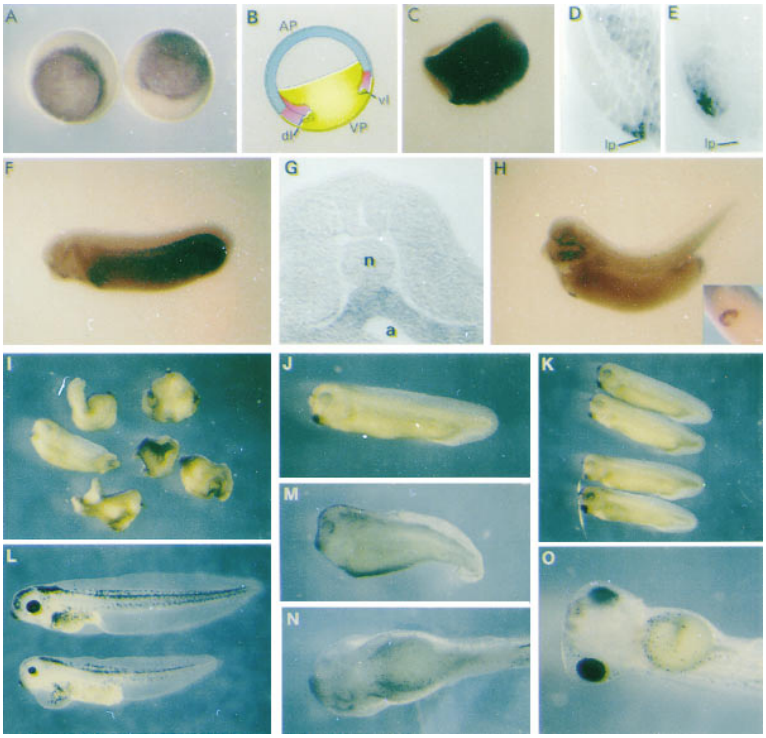

Figure 2. In Situ Hybridizations to Xenopus Embryos

(A) Hybridization of *Xsox17*β to stage 10.5 gastrulae (uncleared), showing hybridization throughout the vegetal pole and superficially around the blastopore.

(B) Fate map of the early gastrula. AP and VP, animal and vegetal poles; dl and vl, dorsal and ventral blastopore lips; blue, ectoderm; red, mesoderm; yellow, endoderm (Keller, 1991).

(C) shows an optical section through a cleared embryo as in (A). It is possible to see hybridization throughout the presumptive endoderm, but the mesoderm and ectoderm are unstained.

(D) This is confirmed by a section of the mesodermal region; lp, blastopore lip.

(E) For comparison, *Xbra* hybridization is shown; *Xbra* expression ceases when the cells have rolled over the lip.

(F and G) Hybridization of *Xsox17*α to a stage 26 embryo (F); shown in section in (G).

(H) Hybridization of *Xsox17*α to a cleared stage 35 embryo. At this stage, there is staining in the posterior gut and in an annulus where the hepatic diverticulum opens into the submesodermal space; this is where the gall bladder develops (Nieuwkoop and Faber, 1994). The annular appearance of hybridization in the liver region is shown by the inset uncleared embryo.

(I–K) Rescue of *Xsox17*β::*En*^{R} by *Xsox-17*β mRNA. Embryos are shown at stage 33. (I) Embryos injected at the 2-cell stage with 240pg *Xsox17*β::*En*^{R} mRNA. (J) Control embryo. (K) *Xsox17*β::*En*^{R} mRNA (240 pg) plus 60 pg *Xsox17*β mRNA.

(L–O) Phenotypic effects of mRNAs. (L) Effect of *Xsox17*α mRNA. At the top is a stage 42 control embryo, at the bottom is one injected with 120 pg *Xsox17*α mRNA. (M) Stage 33 embryo injected with 120 pg *Xsox17*β::*En*^{R} mRNA. A control at this stage is shown in (J). (N) 120 pg *Xsox17*β::*En*^{R} mRNA. Note the reduced gut coiling compared to a control (O).

the submesodermal space (Figure 2H), a region including precursors of the gall bladder (Nieuwkoop and Faber, 1994).

Probes lacking the HMG domain of *Xsox17*α and small probes containing mostly 3′UTR of both genes gave the same results, except that the UTR probes gave fainter signals (data not shown). Dot blots confirmed the lack of hybridization between the two *Sox17* genes for all probes used (data not shown). We therefore concluded that the probes used in Figure 2 were not cross-hybridizing with each other.

Induction of *Xsox17* Genes by Growth Factors

Animal caps treated with signaling molecules provide a model for events that happen in the normal embryo. Since it has been shown that activin is capable of inducing animal cap cells to adopt an endodermal fate (see Introduction), the fate of the tissues in which *Xsox17*α and -β are expressed, we tested the ability of growth factors to induce *Xsox17* transcription. Figure 3C shows that activin produces a strong induction of both *Xsox17*α and -β, with a higher threshold than for *Xbra*. In contrast, FGF induced neither (Figure 3D).

Overexpression of the *Sox17* Genes Induces Endoderm in Animal Caps

*Xsox17*α and -β mRNAs were transcribed in vitro and injected into the animal pole of both cells at the 2-cell stage, then caps were taken at stage 9 and cultured to various stages. In all of the experiments reported here, the two *Sox17* genes acted identically, as perhaps expected from their sequence similarity. It can be seen that both *Xsox17*α and -β induce the expression of *Endodermin* in animal caps (Figure 4A). *HNF1*β (LFB3) is also induced; this is another endodermally expressed mRNA of the gastrula (Figure 4A), but it is also expressed in the neuroectoderm from stage 12 and in the pronephros from stage 23 (Demartis et al., 1994). Since neural and mesoderm markers are not induced in caps overexpressing these SOX genes (below and unpublished data), we believe that the induced expression represents the induction of endoderm.

We have tested animal caps overexpressing Xsox17α and -β for the induction of two later endoderm-specific, activin-inducible markers, *IFABP* and *Xlhbox8*; Figure 4B shows that both were induced. IFABP is normally expressed in intestine (Shi and Par Hayes, 1994) and *Xlhbox8* in the developing pancreas and duodenum (Wright et al., 1989).

Mesodermal markers are not induced by Xsox17α and -β at any concentration (*cardiac actin*, Figure 4A; *Xbra* at stage 11, data not shown), nor is the neural marker *N-CAM* or the cement gland marker *Xanf2* (data not shown). Thus, the effect of Xsox17α and -β on animal cap cells is specifically to induce endodermal markers.

A Repressor Derivative of Xsox17β Prevents Induction of Endodermal Markers by Activin

Since activin induces both endoderm and the two *Xsox17* genes and the latter induce endoderm, we tested

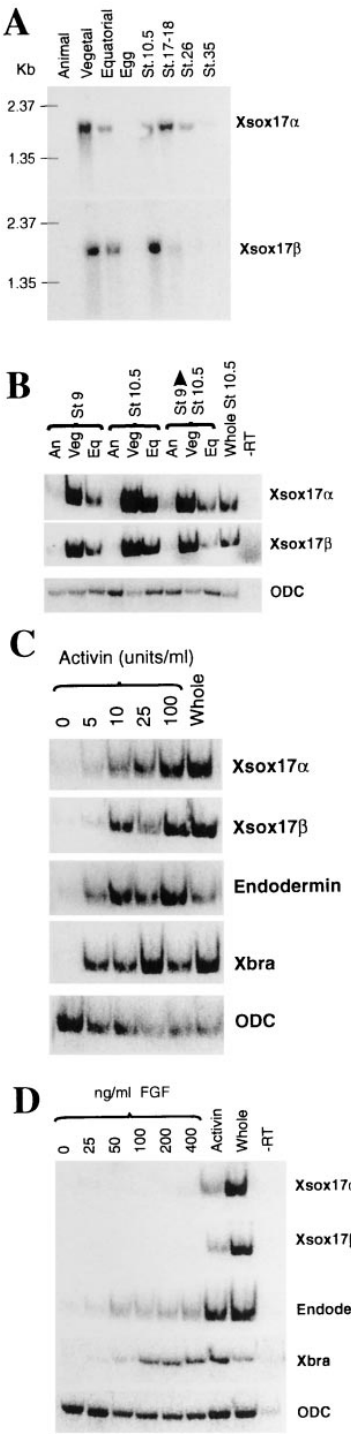

Figure 3. Expression of *Xsox17*α *and* -β

(A) Northern blot of dissected and whole embryos reveals a single transcript of 1.6 kb.

(B) RT-PCR of embryos dissected into animal, vegetal, and equatorial fragments at stage 9 or 10.5. In one experiment, stage 9 fragments incubated to stage 10.5 show maintained low-level *Xsox* expression in the animal cap; this is absent in the caps dissected at stage 10.5. Ornithine decarboxylase mRNA (ODC) is used as a ubiquitous control.

(C and D) Induction of *Xsox17*α and -β by activin (C), but not FGF (D). Animal caps dissected at stage 8.5 and incubated to stage 10.5 and 11, respectively (activin units: Slack et al., 1987).

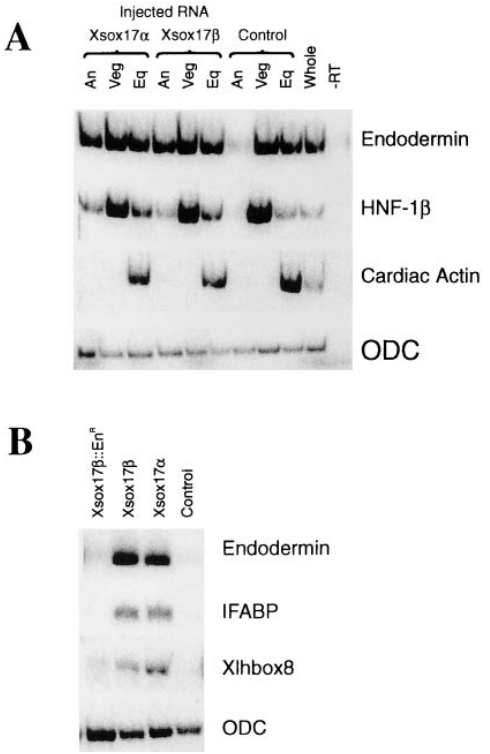

Figure 4. Induction of Endodermal Markers by *Xsox17*α and -β

*Xsox17*α and -β mRNA (300 pg) was bilaterally injected into the animal pole of 2-cell embryos. (A) These were dissected into animal, vegetal, and equatorial regions at stage 9 and incubated to stage 20. (B) Animal caps alone were incubated to stage 35. In the latter, *Xsox17*β*::En*^{R} was injected as a control mRNA.

the hypothesis that *Xsox17*α and -β mediate the action of activin in inducing endoderm. The use of interference-dominant negative constructs to disrupt the function of Xenopus genes, where a genetic knockout is not possible, has been well documented. For transcription factors, a recently described approach to this problem involves use of the Drosophila Engrailed repressor domain (Han and Manley, 1993a and 1993b; John et al., 1995; Smith and Jaynes, 1996). This domain has been shown to function as a transcriptional repressor when fused to a heterologous DNA binding domain in Drosophila, Xenopus, and cultured mammalian cells. Such fusion constructs have also been be used to antagonize the function of the range of transcription factors in the Xenopus embryo with high specificity, including Xbra (Conlon et al., 1996), Eomesodermin (Ryan et al., 1996), Brat/Antipodean/Xombi/VegT (Horb and Thomsen, 1997) and Siamois (Fan and Sokol, 1997).

Mouse Sox17 has been shown to have an activation domain, and the degree of similarity between the mouse and Xenopus genes in this region suggests, but does not prove, that this is true of the latter. The first purpose of our experiments is therefore to show that the Engrailed repressor prevents the induction of endodermal markers by the wild-type *Xsox17* genes. This will imply that the wild-type proteins act in a general way as transcriptional activators (although the mechanism could be complex), but of more practical importance, this will give

means of disrupting the activity of the wild type protein in vivo, that is, achieving a functional knockout.

A region comprising the N-terminal region up to and including the nuclear localization signal of *Xsox17*β was fused in-frame to the Engrailed repressor domain. We have shown that ectopic expression of wild-type Xsox17α and -β in animal caps induces *Endodermin* and *HNF1*β (Figure 4). Expression of Xsox17β::En^R does not induce these genes in animal caps (Figure 5C). Hence, the repressor fusion does not appear to be acting like the wild-type proteins in this assay; rather, it blocks the induction of *Endodermin* by wild-type Xsox17α or -β (Figure 5C). Thus, Xsox17β::En^R can be used to inhibit the action of Xsox17α or -β. The En^R domain alone has no effect (data not shown), as reported previously (Conlon et al., 1996; Ryan et al., 1996).

In order to determine whether the activin induction of *Endodermin* and *HNF1*β is mediated by Xsox17α and -β, animal cap explants were taken at stage 8.5 from embryos overexpressing Xsox17β::En^R, and then these were treated with activin, cultured to stage 11, and analyzed by RT-PCR. The activin induction of *Endodermin* and *HNF1*β was strongly down-regulated by *Xsox17*β*::En^R* (Figure 5A), but mesoderm markers were not affected (see also *Xbra* in Figure 5B).

The absence of inhibition of mesodermal markers, at least at low or moderate *Xsox17*β*::En^R* concentrations, is evidence for the specificity of *Xsox17*β*::En^R*. Further evidence for the specificity is given in Figures 5B and 5C. This shows that the knockout of induction of *Endodermin* and *HNF1*β by both *Xsox17* and activin can be rescued by increasing the relative concentration of either of the wild-type constructs. The rescue is more complete for the activin-induced caps in Figure 5B, presumably because levels of endogenous *Xsox17*α and -β mRNAs were raised by the activin. The specificity of the rescue is shown by the fact that chicken *Sox2* and *-3* do not rescue *Xsox17*β*::En^R* inhibition of induction of *Endodermin* by activin (Figure 5D). In addition, these genes, as well as *Sox11*, do not induce endodermal markers in the absence of activin.

Effects of Xsox17β::En^R on Normal Development of Endoderm

The expression of the later, region-specific endoderm markers *Xlhbox8* and *IFABP* is blocked by Xsox17β::En^R in both isolated vegetal poles and in intact embryos (Figure 6). In contrast, the pan-endodermal marker *Endodermin* is reduced by 60% (Figure 6); this is also true of *Endodermin* and *HNF1*-β in the gastrula, (data not shown). More complete inhibition is achieved only with levels of Xsox17β::En^R that in activin-induced animal caps reduce mesodermal marker expression and therefore might be nonspecific, although it is possible that vegetal poles tolerate higher levels of Xsox17β::En^R because they have very high levels of expression of the wild-type *Xsox17* genes.

Xsox17β::En^R induces expression of both *Xbra* (data not shown) and *cardiac actin* in vegetal poles (Figure 6). Since the latter is as late as stage 32, this suggests that blocking Xsox17 activity causes vegetal cells to switch from endodermal to mesodermal differentiation.

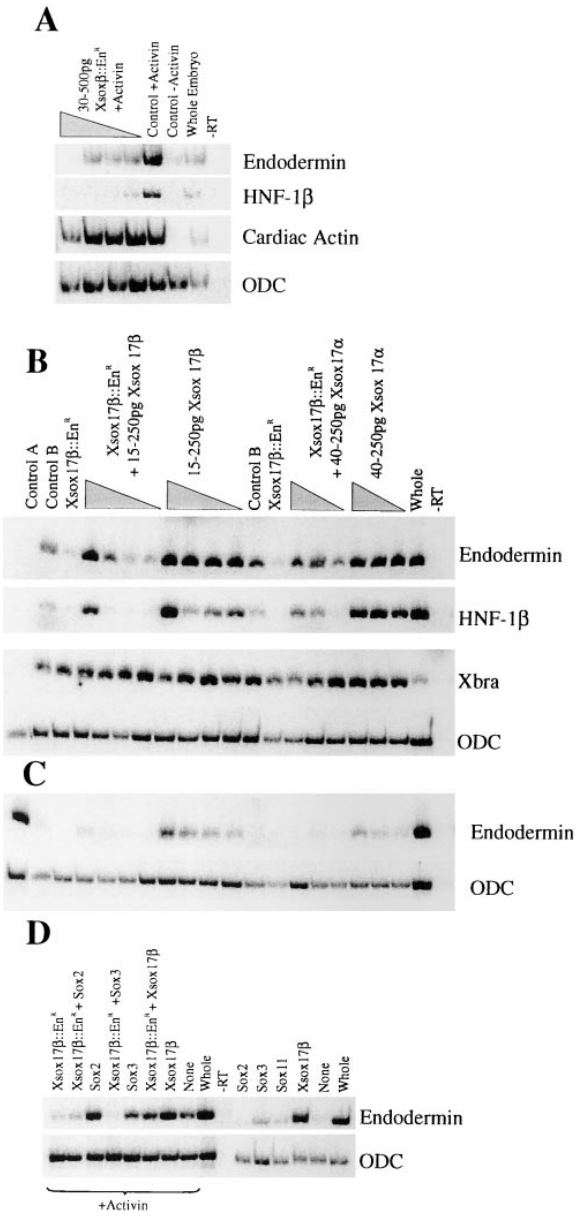

Figure 5. Inhibition of Endodermal Markers by an *Xsox17*β*::En^R* Fusion Construct

(A) Embryos were injected bilaterally with 30, 50, 125, or 500 pg of *Xsox17*β*::En^R*. Animal caps were removed at stage 8.5, treated with activin where shown, and cultured to stage 11, except for those used to detect *cardiac actin*, which were cultured to stage 18. *Xsox17*β*::En^R* (125 pg) inhibited *Endodermin* expression by 86%.

(B and C) *Xsox17*β*::En^R* inhibits endoderm induction by the wild-type *Xsox17* genes and by activin, but this can be rescued by high levels of *Xsox17* mRNA. Embryos were injected at the 2-cell stage with 125 pg of *Xsox17*β*::En^R* and the indicated amounts of *Xsox17*α and -β. Animal caps were dissected at stage 8.5 and incubated to stage 13. In (B), all caps were treated with activin except the uninjected control A; the uninjected controls B were incubated with activin. In (C), all caps were incubated in saline alone except the uninjected control A, which was incubated with activin.

(D) Other SOX genes do not rescue *Xsox17*β*::En^R* or induce *Endodermin*. Embryos were injected with 125 pg of *Xsox17*β*::En^R*, as in (B) and with 250 pg of *Xsox17*β or mRNA encoding chicken Sox2, -3, or -11 (Uwanogho et al., 1995). Caps treated with activin were processed at stage 11, and those without at stage 18.

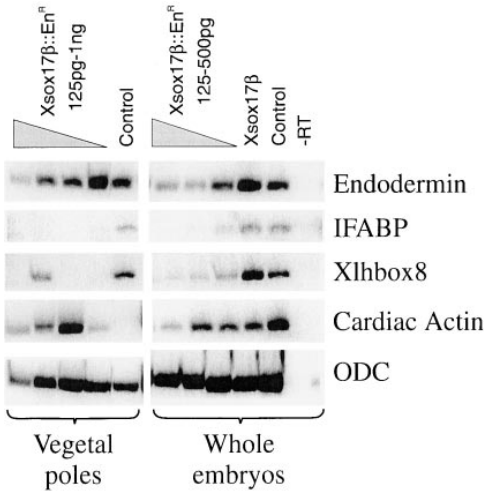

Figure 6. *Xsox17β::En^R* Inhibits Endodermal Markers in Intact Embryos and in Isolated Vegetal Poles

Embryos (2-cell) were bilaterally injected with *Xsox17β::En^R* as shown or 250 pg *Xsox17β* mRNA. Vegetal poles were dissected at stage 9 and incubated to stage 32. Whole embryos were also stage 32. In vegetal poles, *Endodermin* expression was reduced by 64%, *Xlhbox8* by 85%, and *IFABP* by 92%; *Xbra* is increased 6-fold by 125 pg *Xsox17β::En^R*; in whole embryos, 250 pg *Xsox17β::En^R* reduced *Endodermin* by 64%, *Xlhbox8* by 78%, and *IFABP* by 89%.

Taken together, all these data point to a role for endogenous Xsox17α and -β in the differentiation of the endoderm.

Phenotypes Produced by Xsox17α, Xsox17β, and Xsox17β::En^R in Whole Embryos

Bilateral injection of *Xsox17β* or *Xsox17β* mRNA at moderate-to-low concentrations (125 pg/embryo) results in enlargement of the posterior part of the embryo, which becomes more pronounced at higher concentrations, resulting in a bulbous appearance of the posterior endoderm (data not shown). By stage 42, the morphology of the posterior gut is very abnormal (Figure 2L). The other common effect of moderate overexpression is to produce variable abnormalities in head development, but otherwise dorsal axes are relatively normal.

Bilateral injection of high concentrations of *Xsox17β::En^R* mRNA blocks gastrulation just after the formation of the complete blastopore lip, and lower amounts affect the anterior migration of endoderm and mesoderm, and especially the ventral migration of anterior mesendoderm, in a graded dose-dependent fashion (data not shown). The mildest effect is to produce a small subepidermal space in the liver region. The most extreme effects are at levels of *Xsox17β::En^R* that inhibit some, but not all mesodermal markers (*Xbra*, but not *cardiac actin* mRNA induction in animal caps), so the effects on migration could be nonspecific. However, major problems in cell migration would certainly be anticipated if endodermal differentiation were blocked. The lower levels of *Xsox17β::En^R* produce embryos at stage 30–35 with reduced amounts of endoderm posteriorly and thicker endoderm in the anterior, giving the endoderm a pronounced wedge-like appearance (Figure 2M). Later, at stage 41, the elongation and coiling of the gut is greatly reduced (Figure 2, compare N with O).

Even the extreme phenotypic effects generated by 250 pg of *Xsox17β::En^R* mRNA are rescued by coinjection of wild-type Xsox17β (Figures 2I–2K) or a mRNA (data not shown). This is good evidence for the specificity of the repressor construct.

Discussion

We have isolated two closely related SOX genes that, within the accuracy of existing fate maps, are expressed specifically in the presumptive endoderm of Xenopus embryos at the gastrula stage. Since they are both related to murine *Sox17* throughout their amino acid sequence, we have called them *Xsox17α* and -β. It is not clear how far these two genes might differ functionally. While in terms of the main results presented here the two genes behaved similarly, it is possible that they have differing interactions with DNA, and we have noticed some differences in their effects on embryos when overexpressed.

The similarity to murine *Sox17* places the two *Xsox17* genes in a group with *Sox7*, *Sox17*, and *Sox18*, of which *Sox17* and *Sox18* have been shown to have transcriptional activation domains (Hosking et al., 1995; Kanai et al., 1996). Although we have no direct evidence of a transcriptional activation domain in Xsox17α and -β, in addition to the fact that there is sequence homology between the Xenopus proteins and mouse Sox17 in the region identified as having activation function, the fact that the fusion of the Xsox17β HMG domain to the Engrailed repressor domain antagonizes the function of the wild-type proteins in all respects suggests that these proteins do indeed function as transcriptional activators, at least with respect to their role in endoderm formation.

Vegetal cells become determined to form endoderm at gastrulation (Wylie et al., 1987), which is the stage at which we found that *Xsox17α* and -β are expressed throughout the presumptive endoderm. This immediately suggested that these transcription factors are important in initiating endoderm development, a hypothesis strongly supported by the observation that ectopic expression of either causes animal caps, which are specified to form epidermis in isolation, to express endodermal markers. The latter include the general markers *Endodermin* (Sasai et al., 1996) and *HNF1β* (Demartis et al., 1994) at stages when they are endoderm specific (stage 32 and 10.5, respectively), as well as two later markers that are highly region specific. These are *Xlhbox8*, restricted to the developing pancreas and duodenum (Wright et al., 1989) and *IFABP*, found in the intestine (Shi and Par Hayes, 1994; Henry et al., 1996). The presence of this wide range of markers at late stages strongly supports the view that animal caps are induced to form a range of differentiated gut tissues by ectopic expression of *Xsox17α* and -β.

In the gastrula fate map, there is a sharp internal and external boundary between the mesoderm and the endoderm (Figure 2B), which could involve a repression of one tissue type by the other. When Xsox17 is ubiquitously overexpressed, both in the whole embryo and in

activin-induced caps, mesodermal markers are unaffected by Xsox17 expression, implying that mesoderm differentiation overrides Xsox17-dependent endoderm differentiation. It will be interesting to establish the epistatic relationship of *Xsox17* and individual molecules that promote mesodermal development.

To prove the involvement of a gene in a development process, it is necessary to show not only that the gene product can bring this process about, but also that interference with the function of the gene disrupts the process. To make an interference construct for the *Xsox17* genes, we have fused the DNA-binding HMG box of Xsox17β with the repressor domain of Engrailed. This general method has been shown by others to be a highly specific way of repressing transcription factors in developing Xenopus embryos (Conlon et al., 1996; Ryan et al., 1996; Fan and Sokol, 1997; Horb and Thomsen, 1997). Once we showed that this construct eliminates the ability of the Xsox17α and -β to induce *Endodermin* and *HNF1*β, we used it to interfere with endogenous activity of *Xsox17* in three contexts: the activin induction of endoderm, the autonomous development of endoderm in isolated vegetal poles, and in whole embryos.

*Xsox17*β*::En*^{R} prevents the induction of *Endodermin* and *HNF1*β in animal caps by activin, indicating that Xsox17α and -β are essential elements in the induction of endoderm by activin (see Introduction). While activin induces both *Xsox17* genes, *Xsox17*β*::En*^{R} does not prevent this induction by activin of the *Xsox17* genes themselves, but rather blocks the induction of molecules that they themselves can induce without activin. Clearly *Xsox17*β*::En*^{R} acts downstream of the *Xsox17* genes. The specificity of the inhibition is supported by the fact that the induction of mesodermal markers by activin is unaffected.

It is still not at all clear what role induction by molecules of the activin class has in the normal formation of endoderm, although it has been shown that blocking activin signaling inhibits the appearance of *Xlhbox8* but not *IFABP* (Gamer and Wright, 1995; Henry et al., 1996). It was therefore imperative to establish the effect of *Xsox17*β*::En*^{R} on the autonomous appearance of endoderm markers in isolated vegetal poles and on the appearance of these markers in intact embryos. We show that in isolated vegetal poles the appearance of *Endodermin*, *IFABP*, and *Xlhbox8* are all inhibited, the effect on the last two being most complete. *Endodermin* inhibition (like that of *HNF1*-β in the gastrula) is only much greater than 60% when very high levels of *Xsox17*β*::En*^{R} are used; these may have nonspecific effects, although this is hard to establish. The reason for the partial inhibition may be that that we fail to deliver the high concentrations of the repressor to all vegetal cells, but it seems most likely that there is more than one pathway leading to *Endodermin* expression; indeed, it has been reported that chordin and noggin, as well as activin and Vg1, can induce endoderm (Sasai et al., 1996). There is also comparable inhibition of *Endodermin* in late stage swimming tadpoles, but, as in vegetal poles, the region-specific gut markers *IFABP* and *Xlhbox8* are very heavily inhibited. A conservative view is, therefore, that the *Xsox17* genes are obligatory agents in many kinds of gut differentiation but are only contributory in others. *Endodermin* is expressed throughout the gut, but *Xlhbox8* and *IFABP* are in the mid and hind gut, so *Xsox17* genes may play their vital role in the more posterior regions.

The effects of both wild-type and Engrailed repressor proteins on the phenotype of whole embryos are consistent with the role in endoderm formation that we propose. The more moderate effects of Xsox17β::En^R are endoderm specific and involve a reduction in thickness of the hind part of the endoderm at stage 35, followed by inhibition of gut coiling and elongation. As one might expect, the wild-type proteins produce the obverse effects, with the posterior endoderm enlarged.

The specificity of the Engrailed repressor, as well as of the wild-type mRNAs is a critical issue. One line of evidence for specificity is that Xsox17β::En^R and the wild-type genes produce opposite effects in every way. A second is that the latter rescues the repression of endodermal markers by the former, as well as rescuing quite extreme morphological defects that it produces in whole embryos. Last, the wild-type genes do not induce mesoderm markers in animal caps, and conversely Xsox17β::En^R does not repress mesodermal markers in whole embryos or activin-induced caps (except when it is at very high levels), but rather it up-regulates the mesoderm markers *Xbra* and *cardiac actin* in isolated vegetal poles, presumably because cell fate is shifted from endoderm to mesoderm. Of itself, this is strong evidence for a role of the *Xsox17* genes in normal endoderm formation. If endodermal differentiation is inhibited, one would anticipate this change of fate because the vegetal cells are the source of mesodermal inducing signals. It is impossible to prove that the Engrailed fusion does not inhibit some unknown SOX protein, but Sox2, -3, and -11 fail either to induce endodermal markers or to rescue *Xsox17*β*::En*^{R}, which emphasizes the specificity of the effects seen.

Thus, we believe that *Xsox17*α and -β are important regulators of endoderm differentiation in Xenopus. They induce expression of all the endodermal markers tested in animal caps, and *Xsox17*β*::En*^{R} inhibits marker appearance in isolated vegetal poles and activin-induced animal caps. However, these are model systems, and the critical observation is that *Xsox17*β*::En*^{R} also does this in the intact developing embryo. Aside from their clear importance in the development of the endoderm, *Xsox17*α and -β have another role to play experimentally because they are the earliest specific markers of the endoderm, analogous perhaps to *Xbra* or *Eomesodermin* in the mesoderm (Smith et al., 1991; Ryan et al., 1996). Currently several inducers are candidates for forming the endoderm, and there may well also be cell-autonomous molecules involved in specifically initiating endoderm formation, but no candidates for these are known. *Xsox17*α and -β will be invaluable in elucidating these processes.

Experimental Procedures

Biological Materials

Embryos were cultured and dissected by standard methods (Wilson et al., 1986). They were bilaterally injected at the two-cell stage in 3% Ficoll in 0.1× Barths' saline and incubated at 13°C or 18°C. They were transferred to 0.1× Barths' saline at stage 8.5. Dissected fragments for long-term culture were incubated in 0.5× Barths' medium, supplemented with 0.5 mM $CaCl_2$ 10 units/ml gentamycin and

50 units/ml nystatin. Short term cultures were in full-strength Barths' medium.

Construction of Libraries and Subtracted Probe

Poly(A)$^+$ RNA was purified from total RNA extracted (Richardson et al., 1995) from 600 dissected stage 10.5 vegetal poles and 300 whole stage 10.5 embryos using oligo d(T) chromatography. Unidirectional cDNA libraries were constructed using the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene) according to the manufacturer's manual.

To make the subtracted probe, poly(A)$^+$ RNA was purified from total RNA extracted from 600 animal poles and vegetal poles using the Poly-A-Tract system (Promega) according to the manufacturer's protocol. This RNA was used in a subtractive hybridization using the Clontech PCR-Select cDNA Subtraction kit. The protocol recommended was followed with the following changes: all PCRs were carried out with the Expand Long Template PCR system (Boehringer Mannheim), and the primary PCR was carried out with an annealing temperature of 58°C rather than the 68°C recommended. To prepare a probe for screening the libraries, the resulting cDNA mixture was labeled with α-[^{32}P]-dGTP by random hexamer priming.

Library Screening and Sequencing

Libraries were plated to a density of 1–2 $\times$ 10^5 on 20 cm^2 plates. Plaque lifts were carried out with Hybond N$^+$ nylon filters (Amersham) and probed overnight in 0.5 M phosphate buffer with 50 mM EDTA, 7% SDS. Plaques were purified by rescreening of each positive plaque until pure. High stringency (65°C) hybridization was used, with washes at the same temperature for several hours.

The first *Xsox17*β clone was incomplete. Full-length *Xsox17*β was obtained by screening the whole embryo cDNA library with the partial clone. Positives were rescreened by PCR to find the longest insert, using primers to the furthest 5′ region of the partial cDNA and a primer to the T3 primer region of pBluescript.

Sequencing was carried out on both strands using overlapping nested deletions (5′ Exodeletion kit; Pharmacia).

In Situ Hybridization

We used albino embryos from which membranes were removed either manually with forceps, or enzymatically (Islam and Moss, 1996). Embryos were fixed in MEMFA (0.5 M MOPS [pH 7.4], 100 mM EGTA, 1mM $MgSO_4$, 4% formaldehyde), and in situ hybridization was carried out essentially as described by Harland (1991), with the modifications specified by Sasai et al. (1996). Bluescript KS (Stratagene) clones containing the entire cDNA of *Xsox17*α or a partial clone of *Xsox17*β lacking most of the HMG domain were linearized with Xho I and used as a template for transcription using a DIG labeling kit (Boehringer Mannheim). Subsequently, in order to check for specificity, *Xsox17*α, probes lacking the HMG domain were generated by 5′ exonuclease deletion. Short (approximately 400 bp) UTR probes for the transcription reaction were made by exonuclease deletion (5′ Exodeletion kit; Pharmacia) for *Xsox17*α and by linearizing *Xsox17*β with SacII. These probes were dissimilar in sequence.

RNA Analysis

Total RNA was extracted from Xenopus embryos as described previously (Richardson et al., 1995). Northern blots were performed according standard protocols and probed as for library screens. Total RNA from 8 vegetal poles, 4 animal caps, 2 equatorial regions, and two whole embryos was used for each lane in order to equalize rRNA input for dissections.

Quantitative Reverse Transcription-PCR

Total Xenopus mRNA was prepared essentially as described previously (Richardson et al., 1995), but modified slightly for explants. Groups of five animal caps or 5–10 vegetal poles were extracted using 150 μl extraction buffer with 10 μg of glycogen as a carrier. After the first phenol extraction and precipitation step, RNA was resuspended in 60 μl DNase I buffer (20 mM Tris-Cl [pH 8.3], 50 mM NaCl, 2.5 mM $MgCl_2$), containing 20 units DNase I and 10 units of cloned RNase inhibitor (GIBCO–BRL) and incubated for 30 min at 37°C before reextraction.

Quantitative RT-PCR analysis of mRNAs was based on the method of Rupp and Weintraub (1991). RNA (0.5 μg; 1–2 cap equivalents) was denatured at 75°C for 5 min, then cooled on ice. Reverse transcription reactions (30 μl) contained 3.3 μM random hexamers, 3 mM $MgCl_2$, 500 μM dNTPs, 1 unit/μl RNase inhibitor, and 400 units MMLV reverse transcriptase in 1$\times$ PCR buffer (GIBCO–BRL). After incubation for 1 hr at 37°C, reactions were terminated by heating at 95°C for 5 min. PCR reactions in a 25 μl volume used 1 μl of reverse transcription reaction in 1$\times$ PCR buffer with the addition of 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μCi α-[^{32}P]-dGTP, 1 μM each primer, and 0.5 units of Taq DNA polymerase (GIBCO–BRL). Samples were denatured for 3 min at 94°C before cycling through 1 min at the appropriate annealing temperature, 1 min extension at 72°C, and 30 seconds at 94°C. The annealing temperature was 55°C except for *Xlhbox8* (59°C) and *cardiac actin*, when 62°C was used to prevent cross reaction with cytoskeletal actin. Samples were resolved on 6% denaturing polyacrylamide gels containing urea for *ODC* or non-denaturing gels for other markers. Quantification was carried out using a Molecular Dynamics phosphorimager and ImageQuant software.

Cycles (25) were used for primer sets for *HNF1*β, *Xbra*, *Xsox17*α, and *Xsox17*β. For other primer combinations, the number of cycles was as follows: *cardiac actin*, 20 cycles; *Endodermin*, 23 cycles; *IFABP*, 27 cycles; *ODC*, 19 cycles; and *Xlhbox8*, 28 cycles.

All primer sets were tested for linearity of signal, and a linearity curve was run for every marker in every experiment.

Primer sequences for *Xbra* were taken from Wilson and Melton (Melton, 1994), *Endodermin* from Sasai et al. (1996), and for *cardiac actin* from Rupp and Weintraub (1991). Other primer combinations used (5′ primer listed first) were as follows: *ODC*, 5′ GGAGCTGCAAG TTGGAGA 3′, 5′ CTCAGTTGCCAGTGTGGTC 3′ (Bassez et al., 1990); *Xsox17*α, 5′ GGACGAGTGCCAGATGATG 3′, 5′ CTGGCAAGTACAT CTGTCC 3′; *Xsox17*β, 5′ GTCATGGTAGGAGAGAAC 3′, 5′ TCTGTTT AGCCATCACTGG 3′; *Xlhbox8*, 5′AAGGACAGTGGACAGATG 3′, 5′ GGATGAAGTTGGCAGAGG 3′ (Wright et al., 1989); *IFABP*, 5′ GGAA GGTTGACAGAAGTG 3′, 5′ CCAAGAAGTTGGTTGTCC 3′ (Shi and Par Hayes, 1994); *HNF1*β, 5′ GCAGCAGGAACTCCTCAA 3′, 5′ TGGT GGCCATTGGTGAGA 3′ (Demartis et al., 1994).

Construction of Xsox17α and -β, and Xsox17β::En^R Expression Plasmids

Constructs containing the *Xsox17*α or -β open reading frame were generated by PCR amplification of the ORF using Expand High Fidelity polymerase (Boehringer Mannheim). Primers contained BglII sites, and resulting PCR fragments were digested with BglII and cloned into the BglII site of pSPJC2L (Cook et al., 1993).

The *Xsox17*β*::En^R* construct was generated by fusing the N terminal region of *Xsox17*β (a PCR fragment encoding amino acids 1–153) upstream of a fragment encoding amino acids 2–298 of the Drosophila Engrailed protein in the expression plasmid pSPJC2L. This region of Engrailed was amplified from plasmid En^R-pBS (Conlon et al., 1996; a gift of Dr Frank Conlon) using Expand High Fidelity polymerase (Boehringer Mannheim) and included a region encoding a Myc tag at the 3′ end.

Constructs were verified by sequencing. Plasmids were linearized with XhoI to produce a template for transcription. Capped transcripts for injection were generated using the Ambion SP6 mMessage mMachine kit and were tested for translation in oocytes.

The RNA, dissolved in water, was bilaterally injected into the animal pole for experiments on animal caps, or vegetally for isolated vegetal poles. For whole embryo studies, injections were usually vegetal.

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